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TITLE: Investigating the Role of Hepatocyte Nuclear Factor-3
(HNF-3) Alpha and Beta in Prostate Cancer and Cellular
Differentiation

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13. ABSTRACT (Maximum 200 Words) HNF-3 proteins are differentially expressed during mouse prostate development and we demonstrated, using knockout mice, that HNF-3alpha is necessary for normal prostate development. More recently, we showed that HNF-3 proteins are differentially expressed in transgenic mouse prostate cancer models. The aim of this project is to elucidate the role HNF-3 proteins play in prostate cancer cells by employing several molecular biological techniques; in vitro and in vivo models. Western blot analysis showed differential HNF-3 protein expression in human prostate cancer cells. Using tetracycline-regulated siRNAs targeted toward HNF-3alpha, I showed that decreased HNF-3alpha protein levels caused death of PC-3 cells. Overexpression of HNF-3alpha and beta had no effect on the in vitro proliferative index of DU-145 cells, but decreased proliferation of BPH-1 cells. BPH-1 cells overexpressing HNF-3 proteins were chosen for subsequent tissue recombination experiments. These in vivo experiments were performed in December 2004 and tissues will be harvested in the following weeks. We previously showed that HNF-3alpha interacts with the androgen receptor to regulate prostate specific gene transcription. STAT-3 binding sites also exist adjacent to HNF-3alpha on prostate specific promoters and I plan to perform experiments this year to determine how these two proteins interact to regulate prostate gene transcription.					
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DOD PROGRESS REPORT, January 2005

STATEMENT OF WORK

Investigating the Role of Hepatocyte Nuclear Factor-3 (HNF-3) Alpha and Beta in Prostate Cancer and Cellular Differentiation.

Task 1. To infect human prostate epithelial cell lines with HNF-3 Alpha and Beta small interfering RNA (siRNA) retroviral constructs and HNF-3 Alpha and Beta retroviral expression constructs to determine the *in vitro* functional significance of HNF-3 protein down-regulation and overexpression respectively. (1-12 months):

1. Determine the most efficient HNF-3 Alpha and Beta siRNA constructs in PC-3 epithelial cells by northern and western blot analysis. (months 1-2)
2. Establish stable lines of LNCaP, DU-145, PC-3, BPH-1 and primary prostatic epithelial cells which produce tetracycline repressor protein (TetR). (months 1-4)
3. Transfect cells in culture with siRNA or expression constructs, which are driven by a tetracycline responsive derivative of either the human U6 promoter (Tet-U6) or CMV promoter (Tet-CMV) respectively, and select by blasticidin. (months 4-8)
4. Characterize infected epithelial cells following induction by tetracycline incubation. (months 4-12)
 - a. Proliferation (BrdU labeling).
 - b. Apoptosis (TUNEL assay).
 - c. Differentiated function, ie PSA, AR, cytokeratin and chromogranin A expression (western blot and immunohistochemistry).
5. Vary the ratios of HNF-3 Alpha and Beta in epithelial cells and characterize the cells as described above. (months 4-12)

Task 2. To use tissue recombination techniques to determine the *in vivo* functional significance of HNF-3 protein down-regulation and over-expression respectively. (months 12-24):

1. Determine/compare tumorigenicity of two cell lines, carrying stably integrated siRNA or expressing HNF-3 Alpha and Beta, described above by grafting into kidney capsules of athymic mouse hosts. Approximately 48 mice per cell line are required, totaling 96 mice. (months 12-24)
2. Recombine two cell lines with purified urogenital sinus mesenchymal cells to perform tissue recombinant experiments. Approximately 72 mice per cell line are required, totaling 144 mice. (months 12-24)
3. Characterize grafted and recombined transfected epithelial cell lines and conformation of human prostatic cell architecture. (months 12-24)
 - a. Histological characterization.
 - b. Western blot and Immunohistochemical analysis for PSA expression.
 - c. Proliferation and apoptosis quantification.

Task 3. To use molecular biology techniques to determine the interaction of HNF-3 and STAT3 proteins. (months 12-24):

1. Determine protein domain interactions between HNF-3 and STAT3 using immunoprecipitation and GST-pull down assays. (months 6-18)
2. Characterize ARBS/HNF-3/STAT3 complex binding on the PSA promoter using CHIP analysis. (months 18-24)

Key Research Accomplishments

Task 1.

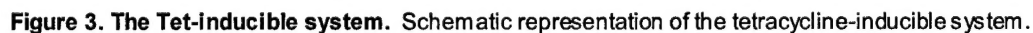
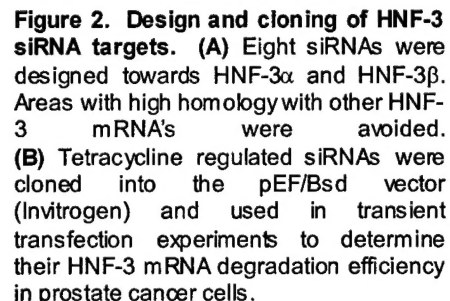
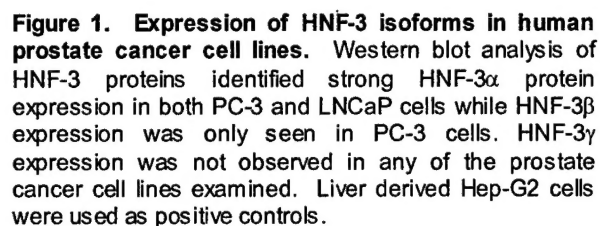
- Western blot analysis identified differential expression of HNF-3 α and HNF-3 β proteins in various prostate cancer cell lines (Figure 1).
- Eight tetracycline regulated siRNAs, designed to target different areas of HNF-3 α and HNF-3 β mRNAs (Figure 2A), have been cloned into the pEF/Bsd vector (Invitrogen, Figure 2B) for use in transient transfection experiments.
- Initial transient transfection experiments, performed on LNCaP and PC-3 cell lines, using siRNAs towards the HNF-3 α mRNA sequence resulted in death of all cells (results not shown).
- As such, a tetracycline inducible system was established (Figure 3) by stably integrating the pTet-tTS construct into prostate cancer cells to produce TetR.
- TetR expressing PC-3, DU-145 and BPH-1 cells have been produced. Stable integration of the pTet-tTS construct in LNCaP cells proved difficult and was not successful. Subsequently, TetR expressing LNCaP cells were kindly provided by Susan Logan (NYU School of Medicine at VAMC).
- Transient transfection have been performed on PC-3 TetR cells and have demonstrated that: A) the tetracycline inducible system works and, B) the siRNAs decrease HNF-3 α protein expression levels with varying efficiencies and induce cell death (Figure 4).
- Similar transient transfection experiments performed to test siRNAs towards HNF-3 β failed to produce a decrease in protein levels. Experiment will be repeated using Dharmacon RNAs.
- DU-145 and BPH-1 prostate cancer cells lines have been retrovirally infected with pLZRS-CMV-HNF-3 α or HNF-3 β expression vectors to produce HNF-3 overexpressing prostate cancer cells (Figure 5). LNCaPs could not be infected with the HNF-3 β expression construct. As such, a lentiviral expression construct is being created to infect these cells.
- Proliferation assays carried out on DU-145 and BPH-1/HNF-3 α and HNF-3 β cell lines showed a decrease in the proliferation index of BPH-1/HNF-3 cells when compared to BPH-1 control cells (Figure 6). These cells were chosen to perform tissue recombination experiments in Task 2.

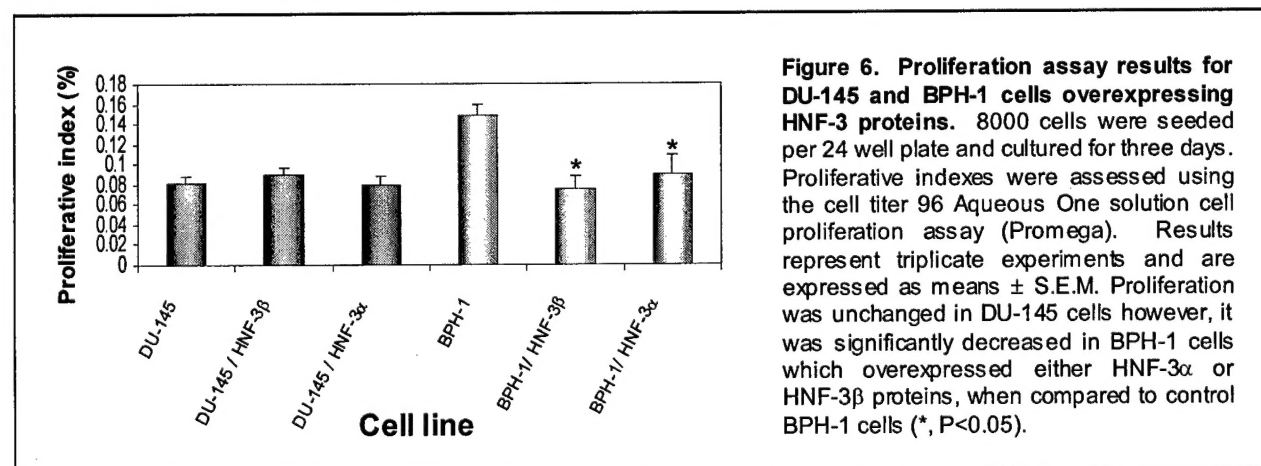
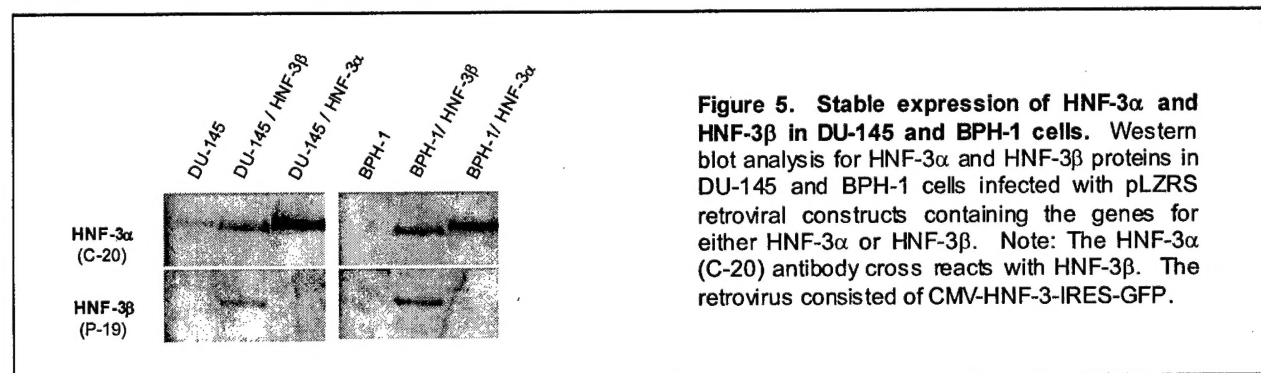
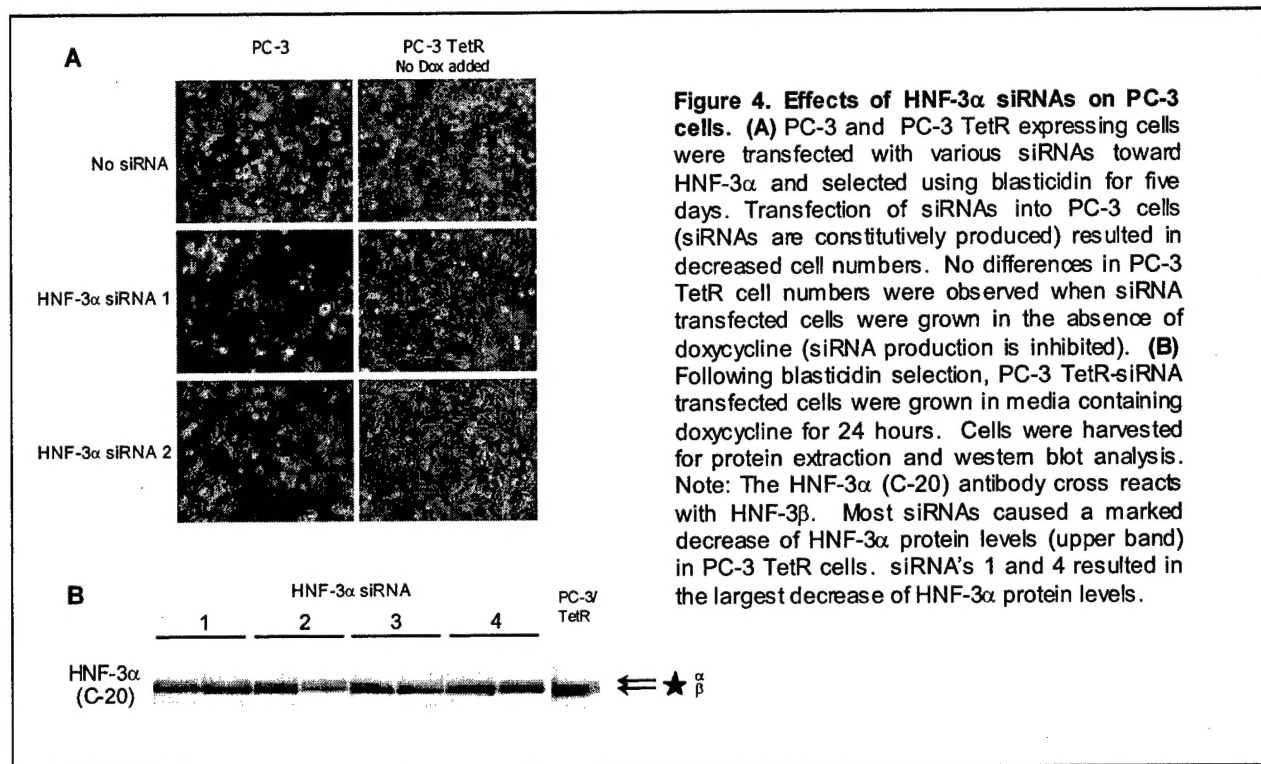
Task 2.

- HNF-3 α or HNF-3 β overexpressing BPH-1 cell lines (Figure 5) have been recombined with rat embryo 18 day urogenital sinus mesenchymal cells and placed under the kidney capsule of athymic nude mice.
- Eight mice have been used for 16 experimental grafts, containing cells over expressing HNF-3 α or HNF-3 β , in the left kidney. Twelve BPH-1 tissue recombination controls have been grafted into the right kidney.
- Mice will be sacrificed at six weeks and three months after implantation, the grafts harvested and analyzed as described in the SOW.

Task 3.

- Sequence analysis, performed on the promoters of numerous prostate specific genes containing known HNF-3 binding sites, identified potential STAT-3 binding sequences (Figure 7A).
- EMSA experiments performed on the PSA and probasin promoter sequences determined that STAT-3 sites were present and adjacent to HNF-3 binding sites (Figure 7B).
- In September I moved from Dr Matusik's laboratory at Vanderbilt University Medical Center in Nashville, Tennessee to Dr Richard Jove's laboratory at The Moffitt Cancer Center and Research Institute in Tampa, Florida. This move will allow collaborations with Dr Matusik and Dr Jove, utilizing their expertise in prostate gene regulation and the STAT-signaling pathway respectively.
- I have acquired various truncated HNF-3 α expression constructs that have previously been used by our laboratory to determine HNF-3 and androgen receptor interactions (Gao *et al.*, 2003). Experiments will be performed with these constructs, along with Dr Jove's various STAT-3 constructs, to determine how STAT-3 interacts with HNF-3 proteins to regulate prostate specific gene transcription.





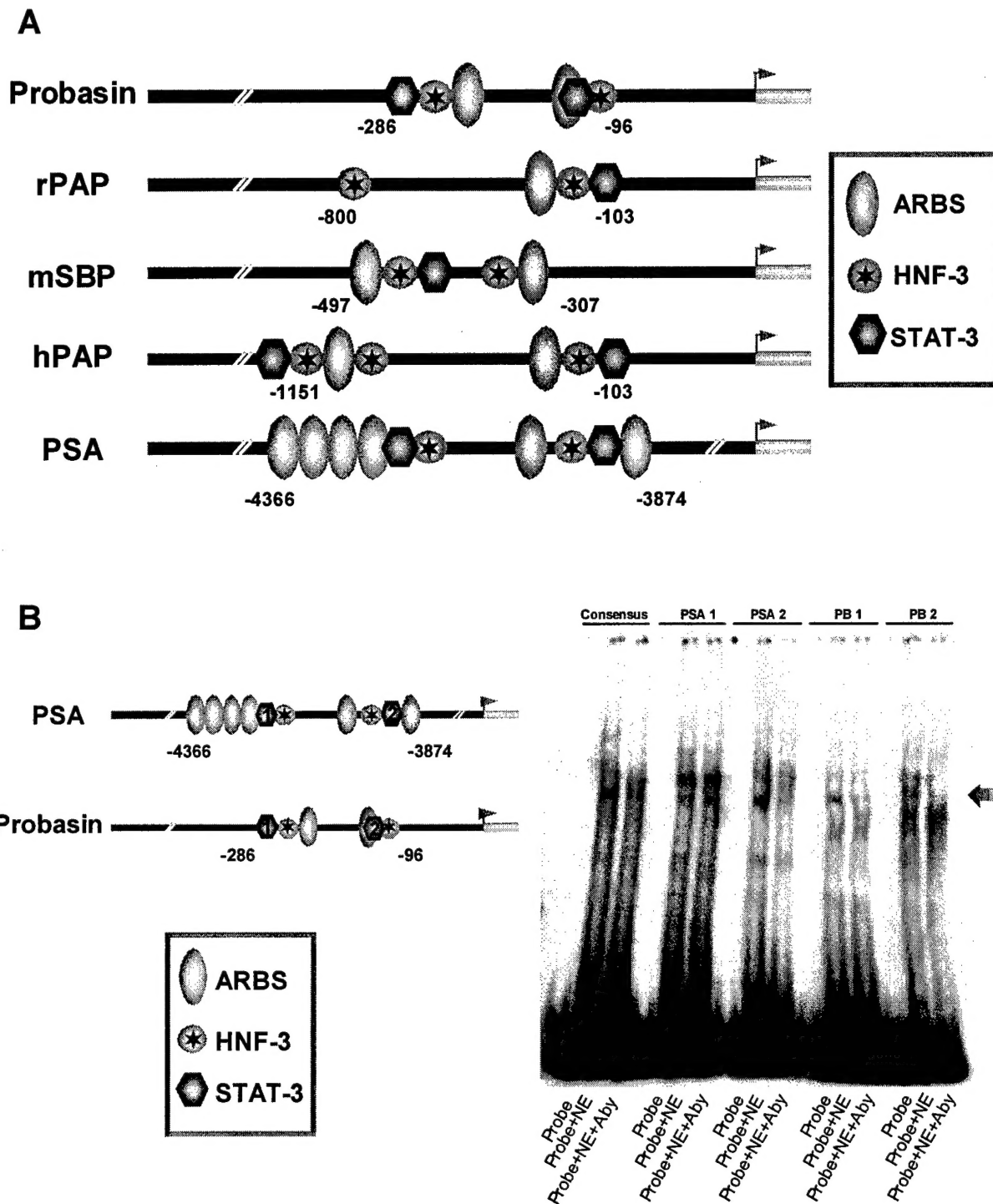


Figure 7. STAT-3 binding sites in prostate specific promoters. (A) Sequence analysis identified potential STAT-3 binding sites adjacent to HNF-3 transcription factor binding sites. **(B)** EMSA analysis confirmed STAT-3 binding in both the PSA and probasin promoters.

Training

Over the last year I have attended and participated in three national meetings and one national workshop:

- Miami 2004 Nature Biotechnology Winter Symposium, Miami, FL, January 2004.
- AACR Edward A. Smuckler Memorial Pathobiology of Cancer Workshop, Snowmass Village, CO, July 2004.
- 10th Prouts Neck Prostate Cancer Meeting, Prouts Neck, ME, November 2004.
- Society for Basic Urologic Research Fall 2004 Meeting, Savannah, GA, December 2004.

I presented posters at all these national meetings/workshops and received travel awards to attend them:

- Society of Basic Urological Research Travel Award, 2004
- 10th Prouts Neck Prostate Cancer Meeting Travel Fellowship, 2004
- AACR Edward A. Smuckler Memorial Pathobiology of Cancer Workshop Member Travel Award, 2004
- Miami Nature Biotechnology Winter Symposium Travel Fellowship, 2004

In addition to presenting a poster, the AACR Pathobiology of Cancer Workshop was a laboratory-based workshop where I attended several round table discussions and also received training in the histopathologic and gross characterization of various human cancers. The Prouts Neck Prostate Cancer Meeting also involved round table discussions with research leaders to discuss and recommend future directions in the field of prostate cancer research.

I was also a participant at the Vanderbilt Ingram Comprehensive Cancer Center (VICCC), Department of Cell and Developmental Biology Retreat, Nashville, TN where I presented a poster. I gave an oral presentation at the VICCC Prostate Cancer Retreat, also in Nashville, TN.

Lunchtime and evening seminars and mini symposiums also contributed to an overall training strategy in place within the VICCC and the department. Finally, laboratory meeting discussions and journal club presentations are integral to the running of the department. I attended and presented within these forums.

Reportable outcomes

Publications

Mirosevich J, Gao N, Gupta A, Shappell SB & Matusik RJ. 2005 The expression and role of Foxa proteins in prostate cancer. *Submitted*.

Gao N, Ishii K, **Mirosevich J**, Kuwajima S, Oppenheimer SR, Roberts RL, Jiang M, Shappell SB, Caprioli RM, Stoffel M, Hayward SW & Matusik RJ. 2005 Arrested prostatic organogenesis due to a failure of cellular determination in HNF-3 α (forkhead box a1)-deficient epithelium. *Submitted*.

Mirosevich J, Gao N & Matusik RJ. 2004 Expression of Foxa transcription factors in the developing and adult murine prostate. *The Prostate*, In Press.

Poster Abstracts

Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R & Matusik RJ. The expression and role of Foxa transcription factor proteins in prostate cancer progression. *Society for Basic Urologic Research Fall 2004 Meeting*, Savannah, GA, December 2004.

Mirosevich J, Gao N, Gupta A, Shappell SB, Jove R & Matusik RJ. The expression and role of Foxa transcription factor proteins in prostate cancer progression. *10th Prouts Neck Prostate Cancer Meeting*, Prouts Neck, ME, November 2004.

Mirosevich J, Gao N, Shappell SB & Matusik RJ. Differential expression of Foxa transcription factor proteins in the transgenic Lady mouse prostate cancer model. *AACR Edward A. Smuckler Memorial Pathobiology of Cancer Workshop*, Snowmass Village, CO, July 2004.

Mirosevich J, Gao N and Matusik RJ. Expression of Foxa transcription factor proteins in the transgenic Lady mouse prostate cancer model. *Miami 2004 Nature Biotechnology Winter Symposium*, Miami, FL, January 2004.

Reagents Generated

Several TetR expressing prostate cancer cell lines, tetracycline regulated siRNAs specific for HNF-3 α and prostate cancer cells lines overexpressing either HNF-3 α or HNF-3 β proteins have been generated from this project. These reagents are currently being used by researchers from the Matusik laboratory and within the department of Interdisciplinary Oncology.

References

Gao, N., Zhang, J., Rao, M. A., Case, T. C., Mirosevich, J., Wang, Y., Jin, R., Gupta, A., Rennie, P. S., and Matusik, R. J. The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and androgen receptor in transcriptional regulation of prostatic genes. *Mol.Endocrinol.*, 17: 1484-1507, 2003.

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